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PRINCIPAL INVESTIGATOR: Christopher P. Evans, M.D.

CONTRACTING ORGANIZATION: University of California
Davis, CA 95616-2075

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Christopher P. Evans, M.D.

E-Mail: cpevans@ucdavis.edu

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14. ABSTRACT

This progress report highlights our past 12 months of work on understanding neuroendocrine differentiation in prostate cancer (CaP). We have made headway into understanding the paracrine relationship between neuropeptide expressing CaP cells and androgen-insensitive CaP cells. Specifically the ability for neuropeptide expressing CaP cells to support the proliferation and migration of the androgen sensitive CaP cells in an environment without androgens (castrate). The more detailed, in vivo experiments will provide a model to look at the relationship in a temporal fashion at the time of castration.

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Introduction

We believe that androgen withdrawal is an event that initiates a cascade promoting the development of androgen independence through NE progression. To date we know of no adjuvant therapies targeting castration initiated molecular events in clinical practice. As such, we seek to better define these early post-castration molecular events. We ***hypothesize*** that a small population of neuropeptide expressing AI CaP cells generated by castration can support the AI survival and growth of androgen sensitive cells in a paracrine fashion. This concept is a novel one regarding the early propagation of CaP following castration. Secondly, we ***hypothesize*** that neuropeptide mediated non-receptor tyrosine-kinase signaling activates androgen regulated genes both through AR and GRP dependent, and AR and GRP independent mechanisms. Demonstration of this concept establishes the rationale for neuropeptide pathway inhibition as singular and combination therapy at the time of castration.

Body

Aim 1. **To determine the paracrine effect of NE cells on androgen sensitive CaP cells.**

a. *Determine the in vitro ability for NE cells to support androgen sensitive CaP cell survival and growth (paracrine effect) in androgen-deprived conditions.* Work on this section not completed yet.

b. *Determine the paracrine effect in soft agar tumorgenesis.* Colony formation of LNCaP-Zeo cells in soft agar assay was promoted when plated chimerically with LNCaP-GRP cells. Due to the paracrine effect of GRP expression from the GRP cells, the androgen sensitive Zeo cells formed twenty-four fold more colonies in androgen-deprived soft agar compared to when growing alone. This stimulation may be partially inhibited by a battery of Src kinase inhibitors, PP2, AZM475271, and AZD0530. Figures of this data appear in the accompanying manuscript.

c. *Determine the paracrine effect on migration in recombinant NE cells.* Stimulation of migration of LNCaP-Zeo cells by GRP cells was assessed by scratch migration assay. This assay was conducted with the help of fluorescence tags and microscopes. LNCaP-Zeo-GFP migrated 1.7 fold more to the scratch region when plated together with LNCaP-GRP-Red cells than alone. MEK1 inhibitor, PD98059 and Src kinase inhibitors, AZM475271 and AZD0530 all partially inhibited this stimulated migration of LNCaP-Zeo-GFP cells.

d. *Study the paracrine effect using the in vivo xenograft model with regard to growth and metastasis.* Co-injection of LNCaP-Zeo cells with LNCaP-GRP cells in castrated SCID mice produced tumors in the prostate regions. The Zeo cells were tagged with green fluorescence protein (GFP) and the GRP with red (Red). Frozen sections of tumor vividly showed patches of green and red colors under the fluorescent microscope. Taken together, both overexpression of GRP may stimulate growth of androgen sensitive Zeo cells both in vitro and in vivo through paracrine effect.

Aim 2. **To evaluate the mechanisms of AR involvement in our NE model.**

a. *Testing of inhibition of neuropeptides, signaling molecules and AR inhibitors individually and in combination on soft agar growth of GRP clones and xenograft cells.* Tumors harvested from GRP implanted mice were re-cultured in vitro to establish xenografts termed as GRP-Pro (derived from Prostate). The expression of human AR, PSA and GRP in tumor xenograft GRP-Pro was analyzed by RT-PCR analysis and supports the authenticity of the clones. Soft agar assay using GRP-Pro showed their aggressive nature as manifested by their androgen- and anchorage- independent growth in 2 weeks (see Figure 5 in attached manuscript). This growth was partially inhibited by the mAb specific to bombesin, 2A11, the androgen inhibitor, bicalutamide, and in combinations (with significant difference $p \leq 0.05$) supporting that the growth is dependent on both the neuropeptide GRP and AR. When synthetic androgen was added with 2A11, the colony formation ability of GRP-Pro resumed to a level similar to control. This further supports the overlapping effect of GRP and AR to the growth of GRP-Pro. Based on the tyrosine kinase display, Src kinase is present in LNCaP cells and involved in signaling via phosphorylation upon bombesin stimulation. Src kinase was constitutively active in LNCaP GRP and its xenograft GRP-Pro when cultured in androgen-free CS serum media. We thus subjected growth of LNCaP GRP-Pro to the inhibitors for Src kinases, AZM475271 from AstraZeneca and PP2. Since MEK1/2 is downstream to Src activation, we also tested the effect of PD98059. Finally, we included the MAPK P38K inhibitor SB203580 because P38 displayed activation in LNCaP cells upon androgen withdrawal. All kinase inhibitors tested decreased the growth 60-80% of control, with significant differences ($p \leq 0.05$). This suggests that the androgen-independent growth of GRP-Pro involves both Src and MEK in a GRP stimulated AR-dependent manner.

b. *Small hairpin RNA (shRNA)-based silencing of NE cells in vitro and in vivo.* No work done yet on this section.

c. *Testing of inhibitory treatments on chimeric tumors in soft agar and in vivo.* Preliminary data only, experiments being repeated.

d. *In vivo testing of inhibitory treatments at different time points.* We are presently developing a Tet inducible GRP LNCaP cell line that can be manipulated to turn the GRP expression on and off via tetracycline. The transfection is complete and clones picked. We are presently testing the clones. Use of an inducible system will permit us to manipulate the paracrine effects temporally and then separate the two cell types by color expression. We are also working out experimental aspects of the flow cytometry for cell separation.

Key Research Accomplishments

We have demonstrated that a paracrine effect exists for androgen insensitive CaP cells to support the survival and proliferation and migration of androgen sensitive CaP cells in a castrated environment. We have validated this in soft agar and in castrated mice. This data will have significant implications for consideration of castration adjuvant therapies.

Reportable Outcomes

Abstract presentations 2004-2005

1. 2004 Yang, J.C., Busby, J.E., Kung, HJ, Evans, C.P. Potent antiproliferative effects of Src kinase inhibition in a model of neuropeptide-induced androgen-independent prostate cancer. European Journal of Cancer 2(8) p.121, No. 405. (NCI/AACR/EORTC joint Molecular Therapeutics in Cancer meeting, Geneva, Switzerland).

2. 2005 Yang, J.C., Busby, J.E., Kung, HJ, Evans, C.P. Src inhibition of neuropeptide-induced androgen-independent prostate cancer. Proceedings of the American Association for Cancer Research, 46: p.748, No. 3180.

3. 2005 Evans, C.P., Busby, J.E., Kung, HJ, Yang, J.C. Androgen-sensitive prostate cancer survival and progression is supported by neuroendocrine prostate cancer cells. Proceedings of the American Association for Cancer Research, 46: p.1033, No. 4369.

4. 2005 Yang, J.C., Busby, J.E., Kung, HJ, Evans, C.P. Src kinase inhibition of neuropeptide-induced androgen-independent prostate cancer. Proceedings of the American Urological Association, 173: p.127, No. 464.

Publications 2004-2005

1. 2005 Yang, J.C., Busby, J.E., OK, J., Novotny, M.J., Borowsky, A.D., Kung, H-J., Evans, C.P. Neuropeptide induced androgen-independent prostate cancer xenograft is mediated through Src tyrosine kinase pathway. Revision submitted to Oncogene.

2. 2004 Busby, J.E., Evans, C.P. Determining variables for repeat prostate biopsy – A review. Prostate Cancer and Prostatic Diseases, 7:93-8.

3. 2004 Penson, D.F., Moul, J.W., Evans, C.P., Doyle, J.J., Gandhi, S., Stern, L, Lamerato, L. The economic burden of metastatic and prostate specific antigen progression in patients with prostate cancer: findings from a retrospective analysis of health plan data. J. Urol., 171:2250-2254.

4. 2004 Evans, C.P. Evidence-based medicine for the urologist. BJU Int., 94:1-2.

5. 2004 Busby, J. E. and Evans, C.P. Old friends, new ways: revisiting extended lymphadenectomy and neoadjuvant chemotherapy to improve outcomes. Curr Opin Urol 14:251-257.

6. 2005 Sam S. Chang, Mitchell C. Benson, Steve Campbell, Juanita Crook, Robert Dreicer, Christopher P. Evans, M. Craig Hall, Celestia Higano, W. Kevin Kelly, Oliver Sartor and Joseph A. Smith, Jr. SOCIETY OF UROLOGIC ONCOLOGY POSITION STATEMENT: REDEFINING THE MANAGEMENT OF HORMONE-REFRACTORY PROSTATE CARCINOMA. Cancer 2005;103:11-21.

7. 2005 Evans, C.P., Fleshner, N., Fitzpatrick, J. and Zlotta, A. An evidence based approach to understanding pharmacological class effect in the management of prostatic diseases. BJU Int. 2005;95:743-749.

8. 2005 Ok, J., Meyers, F. J., Evans, C.P. Palliative care in Urology. J. Urol. in press.

9. 2005 Ok, J., Cambio, A., Lara, P.N., Evans, C.P. Is the use of anything but MVAC justified in the evidence-based medicine era? Curr. Opinion Urol., in press.

10. 2005 McGahan, J. P, Mee R., K., Evans C.P., Ellison, L. Efficacy of Transhepatic Radiofrequency Ablation of Renal Cell Carcinoma. Am. J Radiology. in press.
11. 2005 Cambio, A. J., Evans, C.P. Management Approaches to the Small Renal Tumor.
BJU Int. in press.

Conclusions

We have made headway into understanding the paracrine relationship between neuropeptide expressing, androgen-insensitive CaP cells and their ability to support the proliferation and migration of androgen sensitive CaP cells. The more detailed, in vivo experiments will provide a model to look at the relationship in a temporal fashion at the time of castration.

References

None

Appendices

Attached Manuscript submitted to Oncogene

Neuropeptide induced androgen-independent prostate cancer xenograft is mediated through Src tyrosine kinase pathway[1]

Joy C. Yang, J. Erik Busby, Joon-ha Ok, Michael J. Novotny, Alexander D. Borowsky, Hsing-Jien Kung, and Christopher P. Evans [2].

Departments of Urology [J.C.Y., J.E.B., J.-h.O., M.J.N., and C.P.E.] Biological Chemistry [H.-J.K.] and Cancer Center [H.-J.K., C.P.E.], University of California at Davis, Sacramento, CA 95817, and Center for Comparative Medicine, University of California, Davis, CA 95616 [A.D.B.]

[1]Supported by NIH Grants KO8 DK60748-01, 2RO1 DK/AG52659- 04, and Department of Defense PC10520. Mention of trade name, proprietary product or specific equipment does not constitute a guaranty of warranty by the Department of Defense, nor does it imply approval to the exclusion of other products. The views expressed herein represent those of the authors and do not necessarily represent the position of Department of Defense.

[2]To whom requests of reprints should be addressed, at the Department of Urology, University of California, Davis School of Medicine, 4860 Y St., Suite 3500, Sacramento, CA 95817.

Key words: neuroendocrine, neuropeptides, androgen-independent, xenograph model, prostate cancer

ABSTRACT

Increased number of cells with neuroendocrine differentiation (NED) correlates with advanced prostate cancer (CaP). These cells release neuropeptides including gastrin releasing peptide (GRP) and may facilitate androgen independent transition. Androgen withdrawal induces NED and the release of GRP. We report that the exogenous neuropeptide bombesin increased proliferation and migration in the LNCaP cell line two to four fold respectively. Furthermore, GRP overexpressing LNCaP clones showed enhanced androgen and anchorage independent growth, motility and soft-agar colony formation. Orthotopic GRP cell implantation in castrated nude mice produced significant tumors, with expression of GRP and prostate-specific antigen and androgen receptor (AR) nuclear localization demonstrated by immunohistochemical staining. Soft agar assay of recultured GRP xenografts (GRP-Pro) reconfirmed the aggressive characteristics of the GRP clone. Prevention of GRP-Pro colony formation by MAPK and Src kinase inhibitors suggests their involvement in AR activation. Chromatin immunoprecipitation on LNCaP and GRP clones further supports GRP modulation of AR in the absence of androgen. A specific Src kinase inhibitor, AZD0530 inhibits androgen-independent growth of all GRP and GRP-Pro cell lines, strongly attributing this GRP mediated growth to the Src kinase pathway. This autocrine neuropeptide-expressing xenograft is a useful model to study androgen-independent CaP progression post castration.

INTRODUCTION

Prostate cancer is the most common cancer in American men and the second leading cause of cancer deaths. Removing the androgen stimulus for CaP growth and differentiation initially induces apoptosis; however, CaP eventually loses its dependency on androgens and progresses to a metastatic and androgen-independent (AI) state. A subpopulation of neuroendocrine (NE) cells appears to expand during this transition {Aprikian, 1998 #16}. Withdrawal of androgen from androgen-dependent CaP cells or treatment with stimuli including IL-6, and forskolin promotes acquisition of the NE phenotype through transdifferentiation {Burchardt, 1999 #24;Cox, 1999 #4;Qiu, 1998 #23;Sehgal, 1994 #7}.

NE cells are identified by their neurosecretory granules and expression of neuron specific markers including chromogranin A, neuron-specific enolase and mitogenic peptide hormones such as bombesin/GRP, neurotensin, serotonin, calcitonin, parathyroid hormone-related peptides and others {Abrahamsson, 1999 #1;Heasley, 2001 #17}. AI in CaP patients is shown to correlate well with elevated serum levels of chromogranin A {Wu, 1998 #8}. Elevated expression of GRP receptors in CaP specimens {Bartholdi, 1998 #12;Markwalder, 1999 #13} further supports a role for GRP in CaP progression.

Bombesin/GRP and neurotensin (NT) are soluble ligands which engage heterotrimeric G protein-coupled transmembrane-spanning receptors {Luttrell, 1999 #10}. By binding to their receptors, bombesin/GRP and NT elicit calcium mobilization in PC-3 and DU 145 cells {Aprikian, 1996 #2;Han, 1997 #3} and promote growth and cell invasiveness via proteolytic activities of MMP's in LNCaP and PC-3 cells {Festuccia, 1998 #9;Seethalakshmi, 1997 #6}. We have previously shown that bombesin/GRP can activate AR and support androgen-independent growth in LNCaP

through cell signaling mediated by non-receptor tyrosine kinases such as Etk, Fak, and Src {Lee, 2001 #18}. In vivo androgen withdrawal following establishment of LNCaP tumors results in increased expression of neuroendocrine cells {Burchardt, 1999 #24}. Together, these data suggest that castration induced NED may release soluble factors, which sustain the growth and survival of androgen-deprived cells, eventually leading to the AI state.

In the present paper, we present the epigenetic effect of bombesin/GRP on growth of LNCaP in the androgen-free environment. Addition of exogenous bombesin to LNCaP cells under androgen withdrawal not only supported their growth but also promoted their migratory activity. We further introduced a GRP-expressing vector into LNCaP cells to establish an autocrine NE model. The GRP clones demonstrated enhanced proliferation and migration in androgen-free environments and developed significant tumors in castrated nude mice, providing direct evidence for the neuropeptide's role in androgen-independent growth. Activation of AR by GRP was validated by IHC staining of the mouse tumors for AR and its downstream protein PSA, as well as by chromatin immunoprecipitation (ChIP) assay. The growth of GRP clones and their re-cultured xenografts were inhibited by specific pharmacological inhibitors for Src.

RESULTS

Neuropeptides support androgen-free proliferation and migration of LNCaP cells

The ability of LNCaP cells to proliferate over 72 hours in androgen-deprived charcoal stripped (CS) medium, supplemented with bombesin, neurotensin (100 nM), synthetic androgen (R1881, 10nM), or in regular serum medium were assessed by BrdU proliferation assays. Both bombesin and NT ($p \leq 0.05$) stimulate the growth of LNCaP in

androgen-deprived media 2.5-fold and 4-fold respectively based on the BrdU proliferation assay. The level of NT supplemented growth was comparable to that of R1881 supplementated growth and 20% lower than that of the complete medium (Figure 1A). LNCaP cells are not motile without the aid of basement membrane proteins like fibronectin and laminin. The ability of bombesin to stimulate LNCaP migration was investigated using the Boyden chamber chemotaxis assay. Laminin (50 μg/ml), bombesin (100 nM) and conditioned media (CM) of CS treated LNCaP stimulated LNCaP cell migration by 3.7, 4.2 ($p \leq 0.001$), and 3 ($p \leq 0.001$) folds, respectively, as compared to the negative control BSA only (Figure 1B).

Expression of GRP enhances proliferation and migration of transfected LNCaP cells

Having demonstrated the effects of bombesin on the androgen-independent growth and migration of LNCaP cells, we wished to establish an autocrine model to study the signal pathways involved in androgen independence in vitro and in vivo. To this end, stable LNCaP-GRP transfectants were established by overexpressing GRP cDNA and screened by Northern blotting and RT-PCR (Figure 2A). Positive clones (e.g. GRP1-1, GRP 1-2, GRP4-9 and etc.) were isolated and characterized. These clones behave similarly and the data on GRP 4-9 are described below. GRP 4-9 clone and the parental LNCaP cells were tested for their growth in CS (charcoal-stripped) media by MTT proliferation assay as shown in figure 2B, while the parental LNCaP cells barely grew in CS media, GRP 4-9 proliferated well. In the presence of R1881 or bombesin, both cell lines display a similar growth properties (data not shown). These data support the notion that GRP/bombesin is able to sustain androgen-independent growth of LNCaP. If the androgen independent growth is due to the autocrine release of GRP to the media, we would predict the CM (conditioned medium) of GRP 4-9 has chemotactic effect. Chemotactic migration of

LNCaP-Zeo (LNCaP cells transfected with vector alone) was tested by CM from LNCaP or from GRP 4-9. As expected, LNCaP-Zeo can be stimulated to migrate by bombesinl the positive control, but no by ctl CM, the conditioned media collected from regularly grown LNCaP cells (Figure 2C). The CM from GRP cells (GRP CM), however, stimulated the migration of LNCaP-Zeo by more than 3-fold and this stimulation could be reduced by the addition of the GRP specific antibody 2A11 in the lower chamber, suggesting GRP was involved. Migration of GRP 4-9 to the ctl CM was more than that of the LNCaP-Zeo line, reflecting the random chemokinetic migration of the cells simulated by the GRP autocrine loop. The number of cells migrating through the nucleopore membrane was two-fold greater than that of LNCaP-Zeo, and it could only be inhibited slightly by 2A11 added to the upper chamber. On the other hand, migration of GRP 4-9 was further stimulated by CM from GRP cells by more than 2-fold greater than by ctl CM. This chemotactic migration however could be inhibited only by addition of the GRP specific antibody 2A11 to the lower chamber.

GRP promotes in vitro and in vivo tumorigenesis in androgen-free environments

The above results showed that GRP autocrine loop confers androgen-independent growth and increased motility of LNCaP. We then asked whether it also induces anchorage-independent growth. The ability of LNCaP-Zeo and GRP 4-9 to confer anchorage-independent growth was compared by the soft agar colony formation assay. GRP 4-9 produced significantly more (25x) colonies than LNCaP-Zeo in CS medium, suggesting that the overexpression of GRP induces both androgen- and anchorage-independent growth (Figure 3A). The GRP specific antibody, 2A11 and the anti-androgen, bicalutamide partially inhibited this effect, suggesting GRP and its activation of androgen receptor are involved in this process. A prediction of the above experiment is that

LNCaP GRP clone should be tumorigenic and the tumor should grow in an androgen independent manner. When the LNCaP GRP 4-9 clone was orthotopically implanted into prostates of castrated male nude mice, 8 of 12 mice grew tumors from clone 4-9 cells, whereas no mice (0 out of 4) receiving LNCaP-Zeo cells displayed any sign of tumor growth (Figure 3B). To generalize the finding, LNCaP GRP 1-1 was also implanted in a similar manner and 4 of 5 produced tumors. H & E staining of the tumors showed characteristic human CaP tumors adjacent to mouse prostate tissue. Detection of GRP by anti-GRP IHC staining was compared in normal tissue and GRP 4-9 tumors. Only minimal patchy staining of GRP was detected in the control mouse prostate epithelium, but GRP was evident throughout the cytoplasm of GRP 4-9 tumors (Figure 4A). Staining with anti-human AR antibody further revealed its location in the nuclear region of GRP 4-9 tumors, suggesting translocation of AR into the nucleus, and indication of its activation by GRP (Figure 4B). The AR-regulated expression of PSA was clearly evident in the tumor specimens (Figure 4C), again supporting the activation of AR by GRP. Mean serum PSA levels in tumor bearing LNCaP-GRP mice was 110.9±14 ng/ml, as compared to 9.5±5 ng/ml in LNCaP-Zeo mice.

Tumors harvested from GRP implanted mice were re-cultured in vitro to establish xenografts termed as GRP-Pro (derived from Prostate). The expression of human AR, PSA and GRP in tumor xenograft GRP-Pro was analyzed by RT-PCR analysis and supports the authenticity of the clones (data not shown). Soft agar assay using GRP-Pro showed their aggressive nature as manifested by their androgen- and anchorage-independent growth in 2 weeks (Figure 5). This growth was partially inhibited by the mAb specific to bombesin, 2A11, the androgen inhibitor, bicalutamide, and in combinations (with significant difference $p \leq 0.05$) supporting that the growth is

dependent on both the neuropeptide GRP and AR. When synthetic androgen was added with 2A11, the colony formation ability of GRP-Pro resumed to a level similar to control. This further supports the overlapping effect of GRP and AR to the growth of GRP-Pro. Based on the tyrosine kinase display and the findings in the Lee et al. paper {Lee, 2001 #18}, Src kinase is present in LNCaP cells and involved in signaling via phosphorylation upon bombesin stimulation. Src kinase was constitutively active in LNCaP GRP and its xenograft GRP-Pro when cultured in androgen-free CS serum media (data not shown). We thus subjected growth of LNCaP GRP-Pro to the inhibitors for Src kinases, AZM475271 from AstraZeneca and PP2. Since MEK1/2 is downstream to Src activation {Unni, 2004 #66}, we also tested the effect of PD98059. Finally, we included the MAPK P38K inhibitor SB203580 because P38 displayed activation in LNCaP cells upon androgen withdrawal {Lee, 2004 #59}. All kinase inhibitors tested decreased the growth 60-80% of control, with significant differences ($p \leq 0.05$). This suggests that the androgen-independent growth of GRP-Pro involves both Src and MEK in a GRP stimulated AR-dependent manner.

GRP modulates activation of the androgen receptor

After all the data suggesting the GRP mediated AR activation at the cellular level, we then sought to illustrate this activation at the molecular level. Transactivation assay was performed with LNCaP and GRP-Pro cells in androgen-free media using PSA-Luc as the reporter. Expression of PSA-Luc in GRP-Pro is almost 6 fold higher than in LNCaP cells in the absence of androgen (Figure 6A). This suggests GRP secreted from GRP-Pro cells is driving the expression. If GRP can activate AR, we would predict that in GRP-Pro, AR should be recruited to ARE sites such as those found in PSA promoter. We therefore performed the ChIP assay on LNCaP and GRP-Pro cells. AR binding was analyzed by

PCR against both the enhancer and the proximal ARE regions. Results (Figure 6B) show AR binding in LNCaP cells treated with synthetic androgen R1881 preferably to the E site, whereas in GRP-Pro AR binding was preferably to the P site. This result is in agreement with our previous report that chemokines such as IL-8 functions differently from androgen in the recruitment of AR to ARE sites {Lee, 2004 #59}. The former favors the proximal (P) site, whereas the latter favors the enhancer (E) site of the PSA promoter.

The role of Src tyrosine kinase in GRP-mediated androgen-independent growth

Based on our previous observation, bombesin/GRP stimulates LNCaP growth in androgen-independent environments through activation of Fak and Src tyrosine kinases. The soft agar colony formation assay also showed that two specific Src inhibitors, PP2 and AZM475271 prevent colony formation of GRP-Pro cells in CS conditions. To further demonstrate that this androgen-independent growth is mediated through the Src signaling pathway, we conducted proliferation assays on GRP and GRP- Pro lines in CS medium, CS with addition of two specific Src inhibitors from AstraZeneca, AZM475271 and AZD0530. AZD0530 and AZM475271 both drastically inhibited cell growth to 50-70% (Figure 7). We then examined the status of Src in all cell line grown in CS medium. LNCaP parental cells displayed little or no Src phosphorylation after treated with CS serum for 3 days. All GRP clones and their tumor Pro lines showed clear Src phosphorylation when grown in CS medium. This phosphorylation was prevented when cells were treated with the potent Src kinase inhibitor AZD0530 concurrently with androgen withdrawal (Figure 8).

DISCUSSION

Neuropeptides including bombesin/GRP, gastrin, neurotensin, cyolecystokinin, bradykinin, and vasopressin, have been identified to be potent paracrine and autocrine growth factors in human cancers {Cuttitta, 1985 #5;Nagata, 1996 #61;Abrahamsson, 1999 #1;Heasley, 2001 #17;Rozengurt, 2002 #63;Kim, 2002 #62;Guha, 2003 #64}. Especially in prostate cancer, studies have shown that neuropeptides promote cell growth {Aprikian, 1998 #16}, migration, protease expression {Festuccia, 1998 #9}in PC-3 cells, and even androgen-independence in LNCaP cells{Burchardt, 1999 #24;Lee, 2001 #18}. Expression of neutral endopeptidase which cleaves and inactivates neuropeptides inhibits the transactivation of the insulin-like growth factor signaling pathway that leads to tumor cell survival {Sumitomo, 2001 #65}. In our study, we first tested whether exogenous neuropeptides could stimulate LNCaP cell proliferation and migration. Our data showed that bombesin and neurotensin could confer androgen-independent growth in LNCaP to a similar extent compared to the synthetic androgen R1881. Bombesin also greatly promoted the migration of LNCaP cells. The migration-enhancing activity in CM from androgen-deprived LNCaP could be attributed to neurotensin secreted in the media, which could be steadily measured by the neurotensin ELISA assay (data not shown). Taking from here, we hypothesized that the expression of GRP in LNCaP cells may facilitate a more aggressive phenotype via an artificial autocrine stimulation. The engineered LNCaP GRP 4-9 cells indeed demonstrated the androgen-independent growth and superior migration activities than its parental control. Androgen- and anchorage-independent growth of LNCaP GRP 4-9 cells in soft agar further confirmed that expression of GRP upgraded the aggressiveness of LNCaP cells. This autocrine induced activity eventually led to tumorigenesis of LNCaP in castrated mice, an androgen-free

environment. The IHC staining of intracellular hAR and PSA expression in the tumor specimens together with the direct measurement of the serum PSA in mice with or without tumor, strongly support the role of GRP stimulation of AR in the absence of testicular androgens. The presence of adrenal androgens, the other source of androgens, is not sufficient to trigger the translocation of AR or the expression of PSA. These observations build upon those reported by Burchardt and collegues, who demonstrated that androgen withdrawal of established in vivo LNCaP tumors resulted in enrichment of neuroendocrine cells {Burchardt, 1999 #24}. It has been reported that bombesin may collaborate with low-dose androgen to induce AR activation {Dai, 2002 #57}. Herein we demonstrate that this induction may be accomplished by bombesin/GRP alone to transactivate the downstream PSA gene, both in vitro (ChIP assay) and in vivo (tumor IHC). A recent report using a NE mouse prostate allograft also showed NE secretions were sufficient to support androgen-independent growth of LNCaP and PSA expression in vivo {Jin, 2004 #58}.

Neuropeptide signaling pathways generally initiated with ligand binding to the G-protein coupled receptor and induce signaling transduction via rapid mobilization of intracellular calcium {Rozengurt, 1986 #46;Hellmich, 1999 #40;Luttrell, 1999 #10;Rozengurt, 2002 #63}. It was further demonstrated that bombesin may stimulate association of Fak and Src, both non-receptor tyrosine kinases, in Swiss 3T3 cells dependent on functional protein kinase C {Salazar, 1999 #68}. A role for Fak in mediating CaP cell motility has been shown by Aprikian {Aprikian, 1997 #20}. In collaboration with Fak, Etk is involved in integrin signaling and promotes PC-3M migration {Chen, 2001 #21}. The involvement of these three non-receptor tyrosine kinases Fak, Src, and Etk, has been implicated. {Lee, 2001 #18} although the exact

mechanism how bombesin/GRP activates AR to induce AI growth of LNCaP is not clear. Thus neuropeptides modulate cell proliferation and migration through tyrosine kinases, which promote CaP aggressiveness. Addition of the anti-GRP antibody 2A11 and the anti-androgen bicalutamide inhibited migration and colony formation in soft agar, thereby connecting the autocrine control of GRP and activation of AR. Partial inhibition of androgen-independent growth of the re-cultured GRP-Pro cells in soft agar with pharmacological reagents PD98059 (MEK1 inhibitor), AZM475271, AZD0530, and PP2 (all Src inhibitors) suggests involvement or even collaboration of tyrosine kinases and MAP kinases in neuropeptide mediated AR activation. Inhibitor studies support the notion that Src kinase plays a significant role in GRP mediated AR activation. Signaling pathways downstream of Src leading to AR activation still remain to be investigated. While the use of Src inhibitors in vitro has helped define a mechanistic role for Src involvement, in vivo studies using AZD0530 will require further pharmacokinetic definition.

Induction of NED by androgen deprivation, which eventually leads to CaP progression in AI conditions, agrees with what is observed in clinical cases. Our model for neuroendocrine CaP may facilitate the understanding of underlying mechanisms. The results of this study may have application to clinical scenarios, such as at the time of castration or progression to AI. The specific mechanisms activating AR remain to be determined, but the pathways suggested above may usefully be inhibited to slow or prevent the transition to AI.

MATERIALS AND METHODS

Cell culture-LNCaP cells (ATCC, passages 38-43) were kept in RPMI1640 with 5% regular FBS. When stimulated, cells were switched to phenol-red free RPMI1640 with 5% charcoal-stripped androgen-free (CS) serum.

Proliferation assays-Cell proliferation in various conditions over 72 hours or up to 8 days was studied. Cells were grown in CS medium alone or supplemented with 100 nM of bombesin, neurotensin or 10 nM of R1881. Assays were performed using cell proliferation ELISA, BrdU kit (Roche) or MTT assay.

Chemotaxis migration assay-Migration assays were performed in a Boyden chamber with 8 μm Nucleopore membrane coated with human plasma fibronection (50 μg/ml). 2×10^4 LNCaP cells were placed in the upper chambers, with BSA, laminin (50 μg/ml), bombesin (100 nM), or CS CM in the lower as the attractants, and the chamber was incubated at 37°C for 4 hours to allow cell migration. At the end of incubation, the membrane was stained by Diff-Quik Stain Kit and mounted on a microscopic slide. The non-migratory cells on the upper face were removed and only the cells that migrated through the pores were counted. The entire field was counted under the microscope and each experiment was performed in triplicates.

GRP-expressing construct and transfection-GRP cDNA was amplified from the small cell lung carcinoma DMS53 cell line (ATCC), which expresses GRP. The amplified cDNA was inserted into mammalian expression vector pcDNA 3.1 with selective marker for the antibiotic Zeocin. LNCaP cells were transfected with this GRP construct or the vector pcDNA 3.1-Zeocin only. Stable transfectants were selected with Zeocin (100μg/ml) and they are designated as GRP or Zeo for the GRP overexpression or the

mock transfectants, respectively. The presence of the GRP gene in selected clones was confirmed by Northern blotting and RT-PCR.

Soft Agar Assay-The ability of LNCaP-GRP clones to be tumorigenic in vitro was assessed by soft agar assay. 2 x 10^4 cells were plated in the midst of 0.3% agar. CS medium was used as the sole growth medium. Monoclonal antibody to bombesin/GRP, 2A11 (1 μg/ml, courtesy of Dr. Frank Cuttitta) and anti-androgen bicalutamide (10 μM) were added in two groups of plates. The number of colonies formed was counted after 4 weeks of incubation. Other treatments used were MEK1 inhibitor PD98059 (10 μM), Src inhibitors AZM475271 (Astra-Zeneca, 5 μM) and PP2 (10 μM), and P38MAPK inhibitor SB 203580 (10 μM).

In vivo tumor biology-Two million LNCaP-Zeo and –GRP cells co-suspended with 30% matrigel were injected orthotopically into the castrated male mice, 4 per group. At the end of 4 months, mice were sacrificed and their prostates were harvested to assess the growth of tumor and expression of GRP, AR, and PSA using IHC staining. Formalin-fixed prostate tissue, normal and tumor sections, were embedded in paraffin, sectioned, and stained with antibodies for GRP (Peninsula Laboratories rabbit polyclonal, RGG7130), AR (Uptate rabbit polyclonal, PG21) or PSA (Dako mouse monoclonal, ER-PR8) and detected using the DAKO Envision+ Kit.

Tumors extracted from castrated nude mice were washed, minced, treated with collagenase, and the singe cell suspensions were plated in RPMI1640 with 5% CS FBS medium to establish xenografts. The derived LNCaP GRP sublines termed GRP-Pro were pooled together and subjected to soft agar assay to examine their androgen-independent and tumorigenic characteristics. Synthetic androgen R1881 (1 μM), anti-bombesin MAb 2A11 (1 mg/ml), and bicalutamide (5 μM) were added in the top agar

portion alone or in combinations. The assay was also treated with various inhibitors available for Src tyrosine kinase, AZM457271 (5 μM) and PP2 (10 μM) and MAPK's PD98059 (10 μM) and SB 203580 (10μM).

Chromatin Immunoprecipitation- LNCaP, GRP, and LNCaP-Pro cells grown to sub-confluency were switched to the androgen-free medium for 3 days. One set of LNCaP cells were stimulated with R1881 the night before harvest. Cells were immersed in 1% formaldehyde solution for 10 min for cross-linking, and then were washed in ice-cold PBS, scraped and pelleted. Cell pelletes were resuspended in ice-cold lysis-sonication buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, proteinase inhibitor cocktails) and sonicated with a sonic dismembrator (Fisher Sci. model 550) four times for a 30-s interval of 0.5-s pulses. Chromatin was purified by centrifugation at 15,000 rpm for 20 min. The chromatin solutions were then diluted 1:5 in dilution buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, proteinase inhibitor cocktail) and 200 μl was removed for input control. Complexes were immunoprecipitated{Lee, 2004 #59;Louie, 2003 #60} with 6 μg of anti-androgen receptor antibody (PG-21, Upstate) at 4°C overnight followed by incubation with 50 μl of agarose A beads for 2 h the next day. The beads were washed twice with buffer containing 20 mM Tris-HCl (pH 8), 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS followed by LNDET buffer (0.25 LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0). The protein-DNA complexes were eluted from beads with 50 μl elution buffer (1% SDS and 1 mM $NaHCO_3$) and the eluates were incubated at 65°C overnight to reverse cross-link. Proteins were digested with proteinase K for 1 h at 45°C and the DNA fragments purified with the QIAquick PCR purification kit (Qiagen). 10% of the ChIP product or 1% of the input control was used in each PCR

reaction. The PCR cycling protocol was 5 min at 94°C, 30 cycles of 30 seconds at 94°C, 1 minute at 58°C, 1 minute at 72°C, followed by a 7-minute extension at 72°C. Primers for the AR enhancer region are: 5'catgttcacattagtacaccttg3' and 5'tctcagatccaggcttgcttac3'; for the proximal ARE region: 5'tcctgagtgctggtgtcttag3' and 5'agccctataaaaccttcattcc3'; and for the intervening region: 5'tcatccactcatcatccagcatc 3' and 5'ggagagcaatagacttgggaaacc3'.

Immunoprecipitation and Western blot-LNCaP, GRP and GRP-Pro cells were subjected to androgen withdrawal for 3 days with or without exposure to the Src kinase inhibitor AZD0530 (5 μM). Cell lysates were collected in IP buffer containing proteinase and phosphatase inhibitors, and incubated with anti-Src antibody (UBI) and subsequently protein G agarose beads for immunoprecipitation. Anti-phospho-Src antibody (Cell Signaling) was used to detect Src phosphorylation after Western blotting analysis. Signals were detected by ECL system (Amersham) followed by exposure to X-ray film.

Statistics-All in vitro experiments were performed in triplicate and subjected to paired t-tests using Statview program.

ACKNOWLEDGEMENTS

We thank Dr. Frank Cuttitta (NIH) for providing the monoclonal antibody to bombesin, 2A11.

REFERENCES

Figure legends

Figure 1. Stimulated proliferation and motility of LNCaP in androgen-free conditions by exogenous neuropeptides. *A*, LNCaP cells were maintained in SF, CS, CS plus bombesin (100 nM, CS+Bomb), neurotensin (100 nM, CS+NT), synthetic androgen R1881 (10 nM, CS+R1881) or regular medium (complete) for 72 hours and their proliferative activities

were assayed by BrdU proliferation assay. *B*, Migration assay, 2 x 10^4 LNCaP cells were placed in the upper chamber and stimulated to migrate through an 8μ nucleopore membrane in response to BSA alone, laminin (LMN), bombesin 100 nM, or CM from CS treated LNCaP (CM). After 4 hr incubation, cells that migrated were fixed, stained and counted. Means of at least triplicate experiments were plotted and bars represent standard error of the mean.

Figure 2. The model of an androgen-independent GRP expressing prostate cancer line, with evidence of enhanced proliferation and migration. *A*, GRP expressing clones were established by stable transfection of the expression vector (shown in diagram). The presence of GRP gene was confirmed by Northern blot and RT-PCR. *B*, GRP 4-9 and LNCaP cells were cultured in CS medium for 8 days. Cell proliferation was measured by MTT assay at indicated time points and plotted to show the growth curve. *C*, Boyden chamber migration assay. 48 hr conditioned media from LNCaP-Zeo (ctlCM) or GRP (GRPCM) cells were used as the chemo-attractants. GRP-specific monoclonal antibody 2A11 (1 μg/ml) was introduced as the inhibitor. Bombesin (100 nM) was the positive control. Migration assay was conducted as described in the Materials and Methods. Means of at least triplicate experiments were plotted and bars represent standard error of the mean.

Figure 3. Androgen-independent in vitro (soft agar assay) and in vivo (nude mice) tumorigenesis. *A*, Soft agar assay was performed as described in Materials and Methods. The experiment has been performed independently at least twice and the error bars represent standard error of the mean. *B*, Example of orthotopic implanted LNCaP-GRP tumor grown in a castrated nude mouse. Above left, whole tumor after 4 months. Above right, another tumor with surrounding structures still intact. Lower right, H+E staining

showed LNCaP-GRP tumor on left side, mouse prostate stroma in the middle, and normal mouse prostate gland on the right.

Figure 4. *A*, IHC staining of GRP in normal and tumor specimens. Expression of GRP was clearly visible in the two tumor samples (lower panel) while only patchy stain was detectable in the normal mouse prostate region (upper panel). *B*, IHC staining of AR in the tumor samples. AR was evenly distributed in the human CaP region in the mouse tumors (arrow). Moreover, staining was condensed and localized to the nuclear region rather than cytoplasm, suggesting translocalization of AR upon stimulation. *C*, IHC staining of PSA in the tumor samples. Human specific PSA was only present in the CaP region in the mouse tumors (right panel) but not in the normal mouse prostate glands (left).

Figure 5. Soft agar assay of the re-cultured GRP-Pro xenograft. Soft agar assay was performed as described in the Materials and Methods. Treatments include *A,* monoclonal antibody to bombesin/GRP, 2A11 (1 mg/ml), anti-androgen bicalutamide (BIC, 5 μM), and synthetic androgen R1881 (1 μM); and *B,* MEK1 inhibitor PD98059 (PD, 10 μM), P38MAPK inhibitor SB 203580 (SB, 10μM), and Src inhibitors AZM457271 (5 μM) and PP2 (10 μM). Significant differences denoted by * for $p \leq 0.05$, when compared to the untreated control.

Figure 6. *A*, Transactivation assay. LNCaP and GRP-Pro cells were plated in CS medium and transfected with a PSA-Luc vector. R1881 (1 μM) was added to some wells 24 hours post transfection and luciferase assay was conducted after another 24 hours. Means of triplicate experiments were plotted and bars represent standard error of the mean. *B,* Chromatin immunoprecipitation. AR binding to both the enhancer and proximal ARE in the PSA promoter was revealed through PCR analysis using ChIP assay

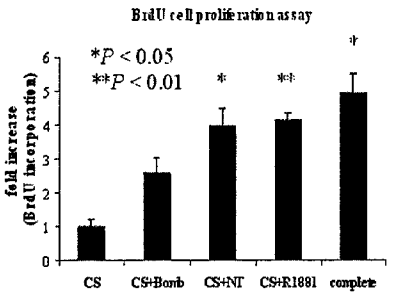
coupled with amplification with primers described in the Materials and Methods. "E", "P", and "I" designate for the upstream enhancer region, proximal ARE region, and the intervening region, respectively.

Figure 7. Cell proliferation assay with Src inhibitors. GRP 4-9 and GRP-Pro cells were plated in CS medium and their growth was monitored by MTT assay over 7 days. Src kinase inhibitors AZD0530 and AZM475271 (5 μM each) were added from day 0. Error bars represented standard error of means.

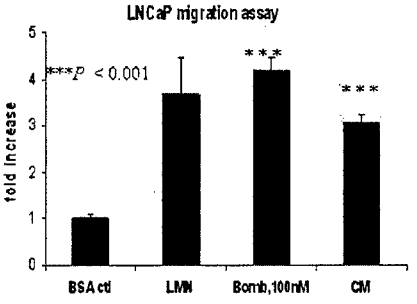
Figure 8. Phosphorylation of Src kinase and its inhibition by AZD0530 in LNCaP parental, GRP and Pro cells. Immunoprecipitation of cell lysates after androgen withdrawal coupled with or without AZD0530 by anti-Src antibody followed by western blotting subsequently probed with anti-phospho-Src. Phosphorylation of Src Kinase was clearly inhibited by AZD0530 in all LNCaP-GRP and –Pro cells.

Figure 1

A


BrdU cell proliferation assay
*P < 0.05
**P < 0.01
fold increase (BrdU incorporation)
0
1
2
3
4
5
6
*
**
*
CS
CS+Bomb
CS+NT
CS+R1881
complete

B


LNCaP migration assay
***P < 0.001
fold increase
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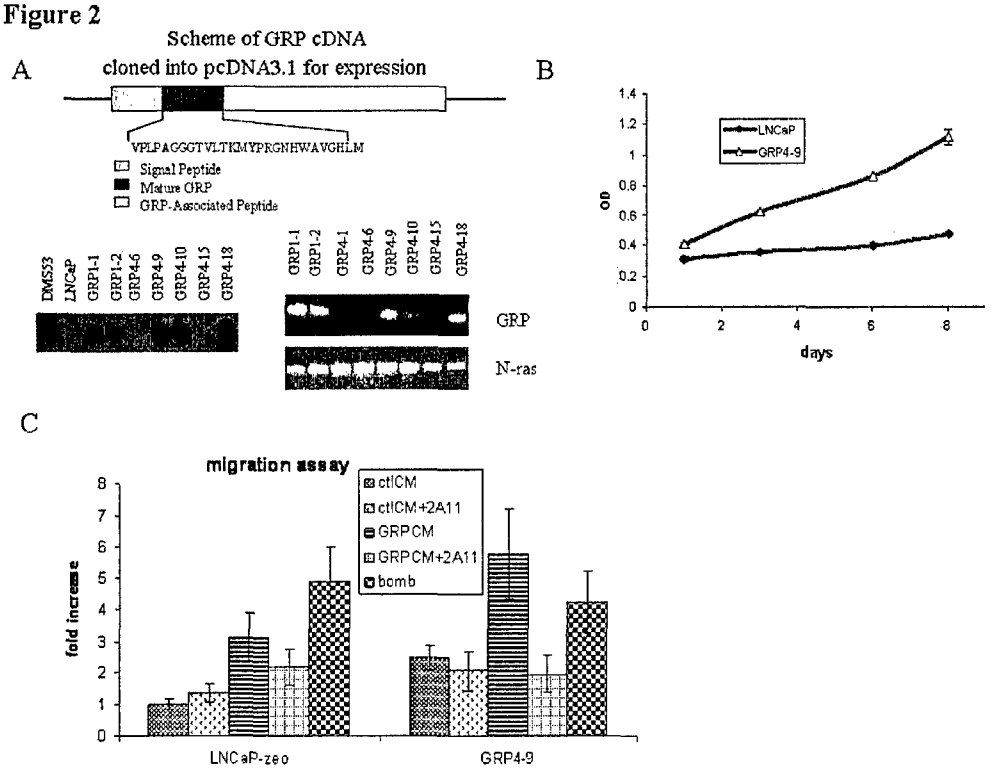
1
2
3
4
5
***
***
***
BSA ctl
LMN
Bomb,100nM
CM

Figure 2


A
Scheme of GRP cDNA
cloned into pcDNA3.1 for expression
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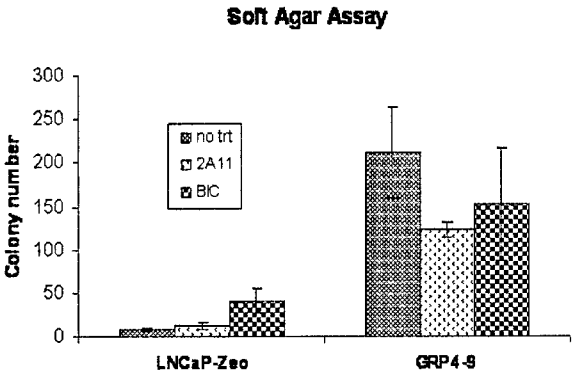
Mature GRP
GRP-Associated Peptide
DMS53
LNCaP
GRP1-1
GRP1-2
GRP4-6
GRP4-9
GRP4-10
GRP4-15
GRP4-18
GRP
N-ras
B
LNCaP
GRP4-9
OD
days
C
migration assay
ctlCM
ctlCM+2A11
GRPCM
GRPCM+2A11
bomb
fold increase
LNCaP-zeo
GRP4-9

Figure 3

A


Soft Agar Assay
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200
150
100
50
0
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2A11
BIC
LNCaP-Zeo
GRP4-9

B

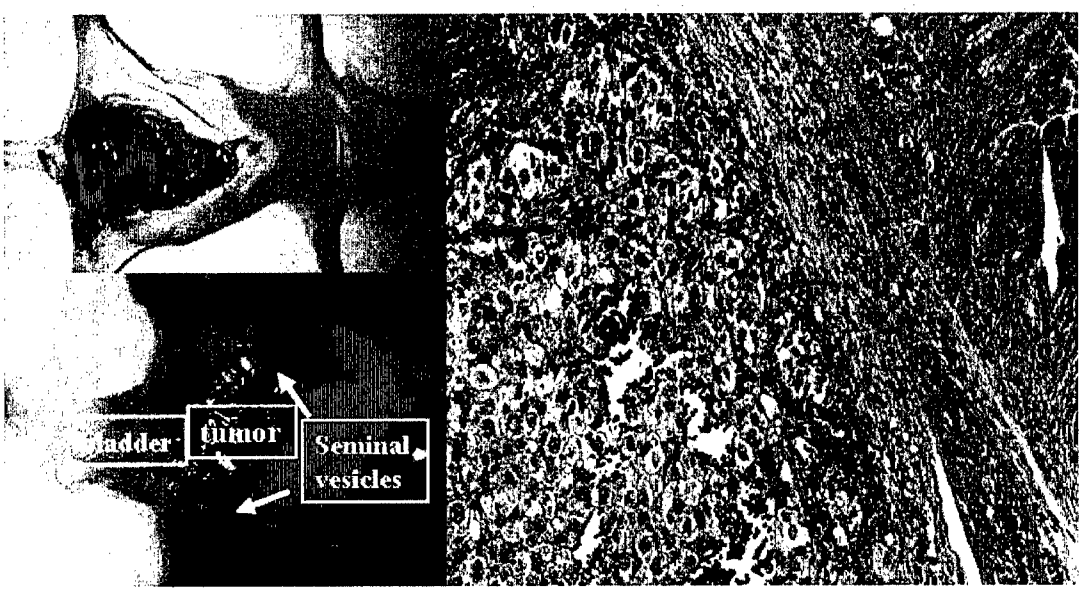
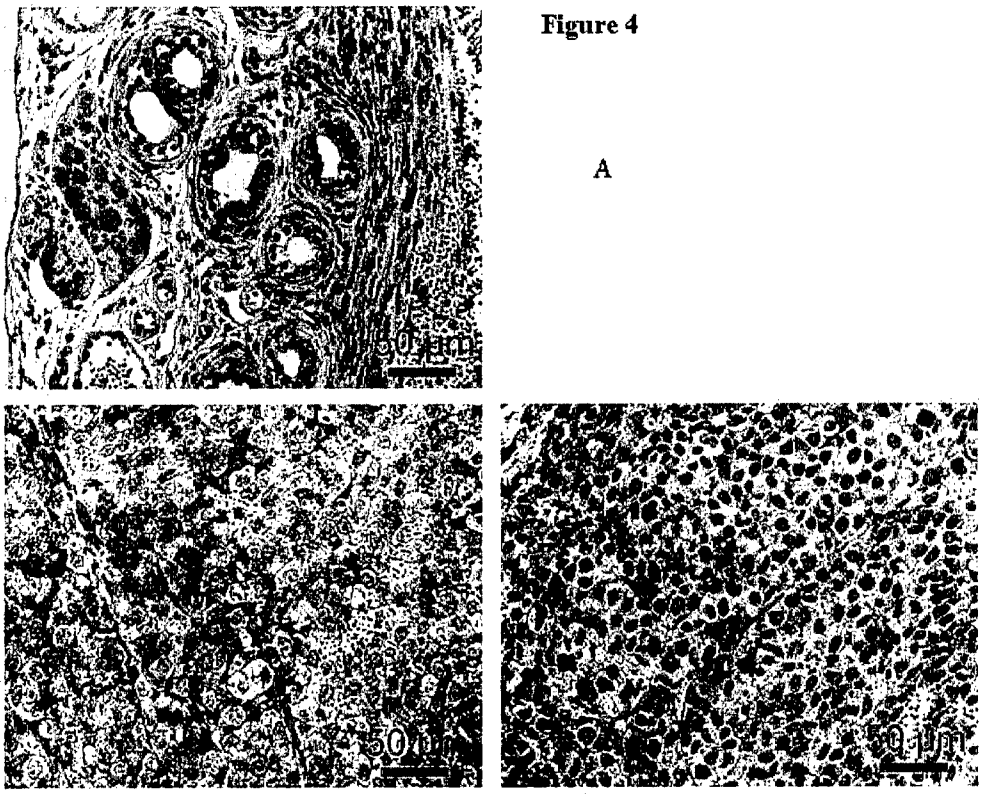

tumor
Seminal vesicles

Figure 4

A



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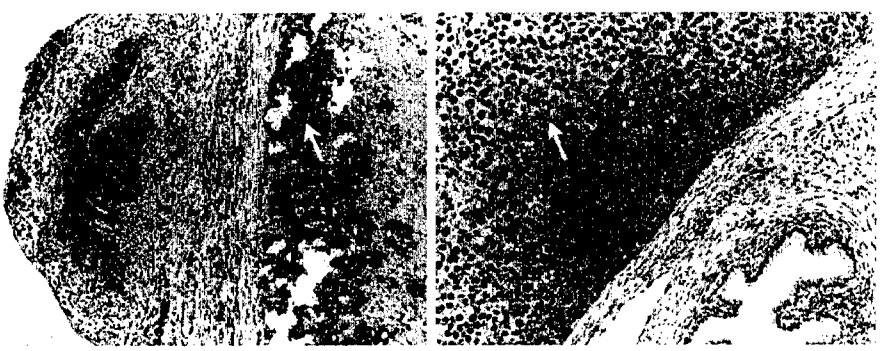

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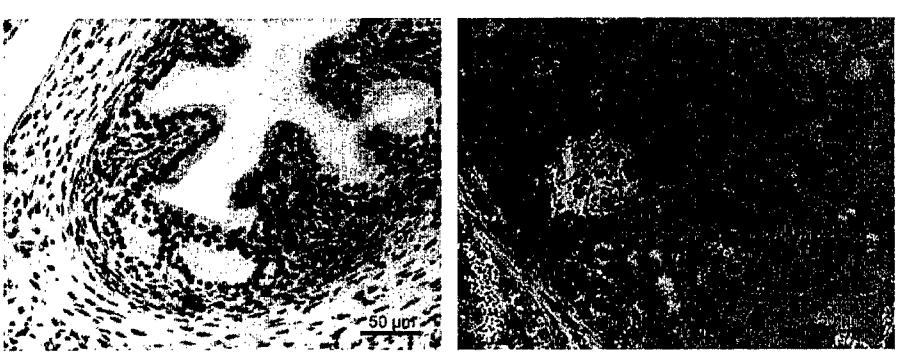

Figure 5

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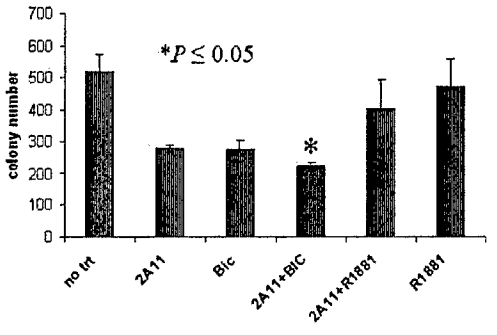
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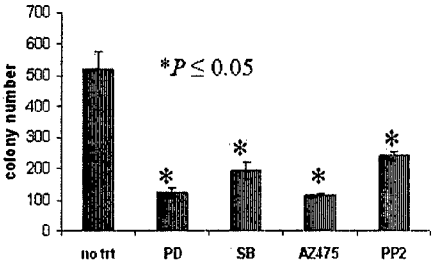
600
500
400
300
200
100
0
colony number
*P ≤ 0.05
*
no trt
2A11
Bic
2A11+BIC
2A11+R1881
R1881

B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500
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200
100
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colony number
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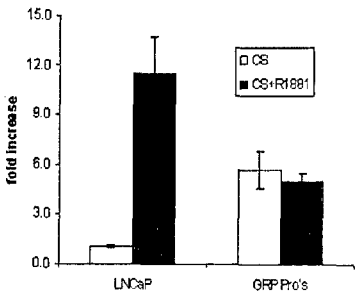
*
*
*
*
no trt
PD
SB
AZ475
PP2

Figure 6

A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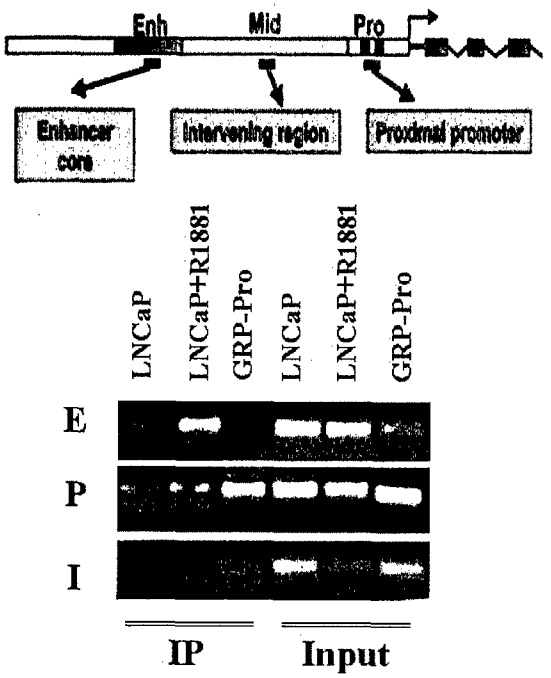
6.0
3.0
0.0
fold increase
CS
CS+R1881
LNCaP
GRP Pro's

B


Enh
Mid
Pro
Enhancer core
Intervening region
Proximal promoter
LNCaP
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GRP-Pro
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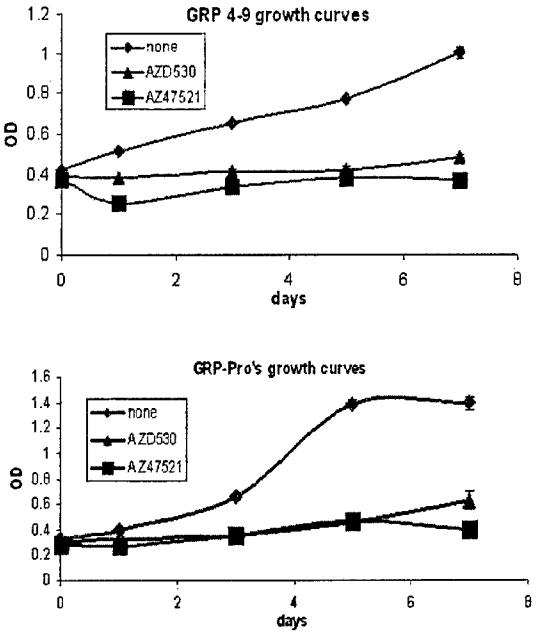
LNCaP+R1881
GRP-Pro
E
P
I
IP
Input

Figure 7


GRP 4-9 growth curves
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AZD530
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OD
days
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1
0.8
0.6
0.4
0.2
0
0
2
4
6
8

GRP-Pro's growth curves
none
AZD530
AZ47521
OD
days
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8

Figure 8